Somatostatin restrains the secretion of glucagon-like peptide-1 and -2 from isolated perfused porcine ileum

LENE HANSEN,1 BOLETTE HARTMANN,1 THUE BISGAARD,1 HITOSHI MINEO,1 PEER N. JØRGENSEN,2 AND JENS J. HOLST1

1Department of Medical Physiology, the Panum Institute, University of Copenhagen, DK-2200 Copenhagen; and 2Novo-Nordisk, DK-2880 Bagsværd, Denmark

Hansen, Lene, Bolette Hartmann, Thue Bisgaard, Hitoshi Mineo, Peer N. Jørgensen, and Jens J. Holst. Somatostatin restrains the secretion of glucagon-like peptide-1 and -2 from isolated perfused porcine ileum. Am J Physiol Endocrinol Metab 280: E1010–E1018, 2000.—Suspecting that paracrine inhibition might influence neuronal regulation of the endocrine L cells, we studied the role of somatostatin (SS) in the regulation of the secretion of the proglucagon-derived hormones glucagon-like peptide-1 and -2 (GLP-1 and GLP-2). This was examined using the isolated perfused porcine ileum stimulated with acetylcholine (ACh, 10−4 M), neurelin C (NC, 10−8 M), and electrical nerve stimulation (NS) with or without α-adrenergic blockade (phentolamine 10−5 M), and perfusion with a high-affinity monoclonal antibody against SS. ACh and NC significantly increased GLP secretion, whereas NS had little effect. SS immunoneutralization increased GLP secretion eight- to ninefold but had little influence on the GLP responses to ACh, NC, and NS. Basal SS secretion (mainly SS28) was unaffected by NS alone. Phentolamine + NS and NC abstract strongly stimulated release mainly of SS14, whereas ACh had little effect. Infused intravascularly, SS14 weakly and SS28 strongly inhibited GLP secretion. We conclude that GLP secretion is tonically inhibited by a local release of SS28 from epithelial paracrine cells, whereas SS14, supposedly derived from enteric neurons, only weakly influences GLP secretion.

paracrine activity; L cells; bombesin; enteric nervous system; gastrointestinal hormones; immunoneutralization

GLUCAGON-LIKE PEPTIDE (GLP)-1 is a peptide hormone secreted from the distal part of the small intestine and the colon in response to meal ingestion (18). It plays an important role as an incretin hormone, stimulating insulin secretion (40), and also functions as one of the hormones of the “ileal brake,” the endocrine inhibition of upper gastrointestinal motility and secretion elicited by the presence of unabsorbed nutrients in the ileum (22, 28, 42). GLP-2 is a peptide hormone derived from the same precursor (1). Much less is known about GLP-2, but recently it has been shown to act as an intestinal growth factor (10). Relatively little is known about the physiological mechanisms that regulate the secretion of GLP-1 and GLP-2, but because of their common precursor, they are supposed to (and also are shown to) be secreted synchronously and in equimolar amounts (29). The presence in the lumen of unabsorbed carbohydrates or lipids provides an important stimulus for GLP-1 secretion (11, 15, 22). However, the GLP-1 response to a meal is usually rapid, with increases occurring within a few minutes after the start of the meal (11, 14, 31), i.e., before the bulk of the meal has reached the lower small intestine. This suggests that neural and/or endocrine factors may also play a role. In humans and pigs, none of the duodenal hormones (gastric inhibitory polypeptide, motilin, cholecystokinin, or secretin) seems to stimulate GLP-1 secretion (27, 28). Furthermore, in humans, neurally induced responses cannot be provoked by modified sham feeding or a cephalic vagal stimulus (32), and, in pigs, electrical stimulation of the mixed intestinal nerves had little effect on GLP-1 secretion in the absence as well as in the presence of α-adrenergic blockers (13). However, somatostatin has been reported to inhibit GLP-1 secretion in sheep (24), as might have been expected in view of the previously demonstrated inhibitory effect of somatostatin on glicentin (or entero glucagon) secretion (4, 6, 7, 23, 35, 39). Like GLP-1 and GLP-2, glicentin is a product of glucagon gene expression in the gut, and the three peptides are secreted in parallel (29). We therefore hypothesized that a stimulatory effect of a given stimulus on the secretion of GLP-1 and GLP-2 might be concealed if the stimulus also caused a local release of somatostatin. If so, the inhibitory effect of somatostatin might outweigh the stimulatory effect of the primary stimulus on GLP-1 and GLP-2 secretion. To study this, we employed somatostatin immunoneutralization (20) in the present series of studies of the neural regulation of GLP-1 and GLP-2 secretion from isolated perfused porcine ileum.

MATERIALS AND METHODS

This study conformed to the Danish legislation governing animal experimentation (1987) and was carried out after permission was granted from the National Superintendence for Experimental Animals.

Perfusion experiments. Young pigs of the YDL-strain weighing 15–20 kg were anesthetized with ketamine chloride (Ketalar, Parke-Davis, Morris Plains, NJ) for premedication, pentobarbital for induction, and chloralose (50 mg/kg) and N2O/O2 for maintenance. After laparotomy, an 80-cm segment of the central ileum, including its arterial and venous supply, was isolated, excised, and positioned floating in Ringer solu-
tion in a perfusion chamber at 37°C. It was perfused via the artery by use of a pulsatile finger pump (Ole Dick, Copenhagen, Denmark) at a constant rate with a Krebs-Ringer-bicarbonate solution containing, in addition, 0.1% human serum albumin (Behringwerke, Marburg, Germany), 5% dextan T 70 (Pharmacia, Uppsala, Sweden), 7 mmol/l glucose, and a mixture of amino acids (Vamin, 14 g/l, Pharmacia) in an amount yielding a total amino acid concentration of 5 mmol/l. The perfusion medium also contained 20% freshly washed bovine erythrocytes (obtained 2–3 days earlier at a local abattoir), and a cyclooxygenase inhibitor (indomethacin, Con- 

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Table 1. Perfusion pressure and motor activity

<table>
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<tr>
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<th>Perfusion Pressure, % of basal</th>
<th>Motor Activity, spikes/5 min</th>
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<tr>
<td></td>
<td>Means ± SE</td>
<td>Changes vs. basal</td>
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<tr>
<td>Basal condition</td>
<td>100</td>
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<tr>
<td>8 Hz</td>
<td>133 ± 10†</td>
<td>P &lt; 0.050</td>
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<tr>
<td>8 Hz + phentolamine</td>
<td>111 ± 3</td>
<td>P &lt; 0.050</td>
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<tr>
<td>8 Hz + phentolamine + somatostatin antibodies</td>
<td>105 ± 1</td>
<td>P &lt; 0.050</td>
</tr>
<tr>
<td>NC + phentolamine</td>
<td>106 ± 1</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>NC + phentolamine + somatostatin antibodies</td>
<td>103 ± 1</td>
<td>ns</td>
</tr>
<tr>
<td>ACh + phentolamine</td>
<td>117 ± 6</td>
<td>P &lt; 0.050</td>
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<tr>
<td>ACh + phentolamine + somatostatin antibodies</td>
<td>114 ± 4</td>
<td>P &lt; 0.050</td>
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8 Hz, nerve stimulation; NC, neumedin C; ns, nonsignificant. *Significantly different from 8 Hz + phentolamine and 8 Hz + phentolamine + somatostatin antibodies, P < 0.050; †significantly different from 8 Hz + phentolamine and 8 Hz + phentolamine + somatostatin antibodies, P < 0.050; ‡significantly different from NC + phentolamine + somatostatin antibodies, P < 0.050.

t-test for paired data. Hormone outputs after NS without and with phentolamine and SS antibodies were compared using ANOVA, whereas hormone outputs after ACh and NC stimulation with and without SS antibodies were compared using a t-test for paired data. Spike frequencies (reflecting motor activity) were recorded for 5-min periods.

RESULTS

The results concerning perfusion pressure and motor activity responses to infusions and nerve stimulations are summarized in Table 1. NS significantly increased perfusion pressure, and phentolamine strongly reduced this response, illustrating successful stimulation of sympathetic efferent fibers (eliciting vasconstriction and therefore increasing perfusion pressure) and the expected effect of α-adrenergic blockade on this response. As expected, NS had little effect on motor activity alone but markedly enhanced motor activity after α-adrenergic blockade, illustrating effective stimulation of nonadrenergic motor neurons under these circumstances (38). Both ACh and NC significantly stimulated motor activity, demonstrating adequate activation of cholinergic and bombesin receptors.

The main finding of the present study is the dramatic increase in GLP-1 and GLP-2 secretion after SS immunoneutralization (Fig. 1). After 4–5 min, both GLP-1 and GLP-2 secretion increased, reaching plateau levels of 813 ± 141 and 995 ± 385% of basal secretion after 15–20 min. In preliminary experiments in which immunoneutralization was carried out without phentolamine, similar increases in GLP-1 secretion were observed (4- to 8-fold increases, n = 3, not shown). Thus the effect of immunoneutralization was independent of the presence of absence of noradrenergic blockade. For GLP-1, plateau values averaged ~400 pmol/l, whereas the GLP-2 plateau amounted to ~800 pmol/l. Because of this unexpected difference, we investigated the molecular nature of the secreted peptides by chromatographic analysis of pooled, concentrated perfusion effluents from four perfusion experiments. The results are shown in Fig. 2. Glucagon (glicentin) immunoreactivity eluted at a distribution coefficient (K_d) of 0.35, corresponding to the elution position of synthetic and natural glicentin. GLP-2 immunoreactivity eluted at K_d 0.55–0.60, regardless of the assay employed (side-viewing or NH2 terminal). The side-viewing GLP-1 assay (code no. 2135) identified a peak at K_d 0.65, and a peak with a similar K_d was obtained with the assay for glycine-extended GLP-1 (code no 92071). The assay for amidated GLP-1 revealed a peak around K_d 0.60.
Fig. 2. Gel filtration profiles of immunoreactive glicentin and GLP-1 and -2 in pools of effluent from isolated perfused ileum collected during somatostatin immunoneutralization. Eluted immunoreactivity is expressed as a percentage of total eluted amount, plotted as a function of the coefficient of distribution (Kd). Values are means ± SE of results from gel filtration of 4 effluent pools. A: results of side-viewing assays for glucagon (×, comprising glicentin and oxyntomodulin) and GLP-1 (●) and the NH₂-terminal assay for GLP-2 ( ▽). B: results of side-viewing assay for GLP-2 ( ▽) and COOH-terminal assays for amidated ( ● ) and glycine-extended GLP-1 ( ● ). In C, total amounts of immunoreactivity eluted as measured in each assay are compared with amount measured with side-viewing GLP-1 assay (code 2135), set to 100%. Significant differences: **P < 0.010; ***P < 0.001. In separate experiments, synthetic glicentin was found to elute at Kd 0.35, oxyntomodulin at 0.75, GLP-2 at 0.55–0.60, and amidated and glycine-extended GLP-1 at 0.60 and 0.65, respectively.

Fig. 3. Effect of nerve stimulation (8 Hz) on secretion of GLP-1 (A), GLP-2 (B), and somatostatin (C) without additions (left), during phentolamine administration (10⁻² M; middle), or during infusion of somatostatin antibodies and phentolamine (10⁻² M) infusion (right). Values are means ± SE of 6 perfusion experiments (n = 3 for GLP-2).
Slightly shifted to the left compared with that of the 92071 assay. Synthetic amidated GLP-1 and glycine-extended GLP-1 both eluted at 0.60–0.65. Measured as total eluted amounts and with the amount identified with the side-viewing GLP-1 assay (code no 2135) set to 100%, both the assay for the amidated and that for the glycine-extended COOH terminus measured significantly less than the side-viewing assay (27.6 ± 4.3%, P < 0.001 for 89390 and 30.6 ± 11.5%, P < 0.010 for 92071; Fig. 2C). However, the sum of the results of these assays (89390 + 92071) did not differ significantly from the amount obtained with the side-viewing assay (P = 0.076). Thus approximately one-half of the total output of GLP-1 was accounted for by glycine-extended GLP-1, explaining the difference between the measured GLP-1 (using antiserum 89390) and GLP-2 plateau values, and possibly also some of the other minor discrepancies between the results of the two assays. There was no significant difference between amounts measured using the side-viewing GLP-1 assay and the assays for GLP-2 or glicentin (Fig. 2C).

Figure 3 shows the results of electrical mixed NS, either alone or in the presence of phentolamine (10⁻⁵ mol/l) and phentolamine + SS antibodies. GLP-1 secretion was unaffected by NS regardless of the perfusion conditions, whereas GLP-2 secretion decreased to a nadir of 52% ± 9% after 3 min of NS. After addition of phentolamine and phentolamine + SS antibody, GLP-2 secretion increased insignificantly during NS to maximally 297 ± 129 and 159 ± 30% of basal, respectively. SS secretion was uninfluenced by NS in the control experiments but increased greatly (to 470 ± 80% of prestimulatory levels, P < 0.010) during phentolamine perfusion. Chromatographic analysis revealed that the SS immunoreactivity released in the basal state mainly consisted of SS28, whereas during NS + phentolamine, mainly SS14 was released (Fig. 4). SS secretion could not be measured during antibody perfusion. Figure 5 shows the NC stimulation experiments during phentolamine alone and during phentolamine + SS antibody perfusion. GLP-1 secretion, expressed in percentage of basal, was significantly enhanced by NC during phentolamine as well as antibody perfusion (to 293 ± 43%, P < 0.010 and 149 ± 16%, P < 0.050, respectively; P < 0.050 for the difference). The absolute output of GLP-1 was larger during NC + antibody perfusion (3.4 ± 0.6 vs. 1.1 ± 0.1 pmol/min, P < 0.050). NC, in all three cases, stimulated GLP-2 secretion under both phentolamine and phentolamine + SS antibody (to 265 ± 42 and 175 ± 52% of prestimulatory levels), but the difference did not reach significance. SS secretion was greatly augmented by NC (to 437 ± 73%, P < 0.010), and chromatographic analysis revealed that SS14 was the predominant molecular form (Fig. 4).

Figure 6 shows the ACh stimulation experiments during phentolamine alone and during phentolamine + SS antibody perfusion. GLP-1 secretion showed a somewhat delayed increase in response to ACh, both during phentolamine and SS antibody perfusion (to 169 ± 12 and 116 ± 4%, in the 5- to 8-min period after start of ACh, P < 0.010 and 0.050, respectively). The absolute outputs of GLP-1 did not differ significantly (0.8 ± 0.2 vs. 1.3 ± 0.4 pmol/min, respectively). ACh also tended to increase GLP-2 secretion under both conditions (to 265 ± 50 and 111 ± 18% of basal secretion, but the changes did not reach statistical significance). ACh did not affect SS secretion.

In the six experiments in which SS14 was infused to a final concentration of 10⁻⁹ mol/l, GLP-1 secretion decreased to 79 ± 7%, P < 0.050. Because of this finding, a dose-response study was carried out. Figure 7 shows the effect of SS28 and SS14 infused to final concentrations of 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/l on GLP-1 and GLP-2 secretion. SS28 dose dependently inhibited both GLP-1 and GLP-2 secretion, reaching 38 ± 10% of basal secretion for GLP-1 and 27 ± 7% for GLP-2 at 10⁻⁶ mol/l (P < 0.01). SS14 had weak and inconsistent inhibitory effects on GLP secretion. During co-infusion of SS28 and NC, GLP-1 secretion was inhibited to 66 ± 5% and GLP-2 secretion to 50 ± 9% (P < 0.05), both significantly different from the stimulated secretion observed with NC alone (P < 0.01). In contrast, during...
Co-infusion of SS14 and NC, both GLP-1 and GLP-2 secretion increased (P = 0.08 and 0.09, respectively) to levels not significantly different from those obtained with NC alone.

Addition of the 2,4,6-trinitrophenyl antibodies had no influence on any registered parameter (data not shown).

**DISCUSSION**

The most important finding of the present study is the dramatic increase in the secretion of GLP-1 and -2 in response to somatostatin immunoneutralization. The antibodies were administered as a highly purified solution of γ-globulins, and there was no effect of
infusion of control antibodies, speaking against unspecific effects. In addition, the high concentrations of the glucagon-like peptide measured during immunoneutralization were verified by chromatographic analysis of the effluent (see further discussion). Furthermore, the inhibitory effect of exogenous somatostatin was confirmed. It must be concluded, therefore, that under the prevailing experimental conditions, local somatostatin secretion exerts a marked restraint of the secretory activity of the L cells, the endocrine cells known to be the source of the glucagon-like peptides. This raises the question of the cellular source of somatostatin involved. In previous studies, we found that the density of endocrine epithelial somatostatin-secreting cells is lower in the ileum compared with the more proximal segments of the gut (41). On the other hand, the ileal mucosa harbors a dense network of somatostatin-containing nerve fibers, which seem to originate in the nerve cell bodies of the submucous plexus (41). The predominant secretory product of the endocrine somatostatin-producing cells is thought to be SS28 (corresponding to the COOH-terminal 28 amino acids of prosomatostatin) (2, 12), whereas the main product of the intestinal neurons is SS14 (the COOH-terminal 14 amino acids of prosomatostatin) (37). In the present study, the chromatographic analysis of ileal effluent revealed that almost all of the immunoreactivity released in the basal state corresponded to SS28. Together, these findings indicate that the source of somatostatin interacting with the L cells in the basal state is the endocrine cells, whereas somatostatin released during nerve stimulation or neumedin C infusion corresponds to SS14, and therefore presumably is released from the enteric neurons. In addition, our experiments showed that SS28 was much more efficacious and potent as an inhibitor of GLP secretion than SS14. We conclude, therefore, that the local somatostatinergic control of GLP secretion is exerted by paracrine cells of the mucosa releasing mainly SS28, which is likely to interact with sstr-5 receptors on the L cells, because sstr-5 is the only somatostatin receptor with a preference for SS28 (33). Brubaker et al. (5) reported that GLP-1 was capable of releasing both SS14 and SS28 from rat intestinal cultures. In preliminary experiments using the experimental model of the present study, in which the morphological integrity of the tissue and the paracrine relationships are preserved, we found a dose-related release of somatostatin upon intravascular infusions with GLP-1, with $10^{-9}$ mol/l being the most effective concentration (unpublished studies). This suggests that GLP-1 and somatostatin secretion may be mutually interdependent in a paracrine relationship, whereby GLP-1 limits its own secretion by activating a SS28-mediated paracrine inhibition.

The possible influence of SS14 released from enteric neurons on GLP secretion was investigated in a series of nerve stimulation experiments. Electrical stimulation of the mixed extrinsic nerves innervating the ileal segment elicited vasoconstriction in the segment, attenuating the efficiency of stimulation. However, this had little effect on the secretion of the GLPs or somatostatin, although a tendency to inhibition was noted. This is in agreement with the results of yet unpublished studies, in which intraarterial norepinephrine inhibited GLP release (Mineo and Holst, unpublished studies). In the present experiments, addition of the $\alpha$-adrenergic blocker phentolamine reduced the hypertensive response as expected (36), but now nerve stimulation strongly stimulated somatostatin release. This suggests that the intestinal somatostatinergic neurons are innervated by inhibitory, noradrenergic fibers as well.

![Fig. 7. Effect of infusions of SS28 and SS14 in final concentrations of 0.1 nM, 1 nM, and 10 nM (left) on GLP-1 secretion (open bars, A) and GLP-2 secretion (solid bars, B). At right, effect of coinfusion of SS28 + NC (GRP) and SS14 + NC, and NC alone, on GLP-1 and GLP-2 secretion. All responses are expressed as % basal secretion. Values are means ± SE of 4 perfusion experiments. Significant differences from basal secretion: *P < 0.050; **P < 0.010; ***P < 0.001.](http://ajpendo.physiology.org/Downloadedfrom)
as strongly stimulatory fibers. Acetylcholine alone had no effect on somatostatin release and is therefore unlikely to act as the stimulatory transmitter; vasoactive intestinal polypeptide, which is released from intrinsic ileal neurons in the pig under similar conditions (25), might be responsible. GLP secretion was slightly augmented by nerve stimulation during α-adrenergic blockade. In addition, acetylcholine weakly but significantly stimulated secretion, in agreement with previous investigations performed in vivo and in other species, suggesting that acetylcholine may stimulate L cell secretion (16, 34, 44). Thus it appears that cholinergic mechanisms may lead to a weak stimulation of GLP-1 secretion.

We then hypothesized that the dramatic release of somatostatin that was observed during α-blockade might dampen the secretory response of the L cells. To test this hypothesis, nerve stimulation was repeated during somatostatin immunoneutralization, with a technique previously demonstrated to bring about a lasting and virtually complete elimination of the actions of exogenous as well as endogenous somatostatin (20). However, under these conditions (which included α-adrenergic blockade), nerve stimulation had no significant effects. Our immediate conclusion would be that somatostatin, released during nerve stimulation, had no influence on the GLP response to nerve stimulation. A similar conclusion was reached from the experiments involving the neurotransmitter neuromedin C. Neuromedin C, the COOH-terminal active fragment of mammalian bombesin or GRP, has been shown to provide a strong stimulus for GLP secretion (29), as also confirmed here. Neuromedin C, however, also stimulated somatostatin secretion, almost as much as nerve stimulation under phentolamine infusion. The GLP response to neuromedin C was preserved during somatostatin immunoneutralization. The fractional increase was much smaller than before antibody infusion, but the absolute amount secreted, and also the amount above that elicited by the immunoneutralization, was larger. Similar results were obtained with acetylcholine. These results would, again, be interpreted to indicate that somatostatin released in response to nerve stimulation had only a minor influence on nerve- or neurotransmitter-stimulated GLP-1 secretion. This notion is supported by the finding of a weak effect of SS14, the molecular form released during nerve stimulation, and by the finding that only SS28, but not SS14, could inhibit neuromedin C-stimulated GLP secretion.

From our experiments, we conclude that paracrine SS28 is a main regulator of GLP secretion and that the extrinsic innervation of the gut exerts a weak, presumably cholinergic stimulatory effect on GLP secretion that is independent of somatostatinergic intestinal neurons. GRP (or neuromedin C) is much more efficacious than acetylcholine, raising the possibility that GRP-producing neurons could be involved in a stimulatory secretory control of GLP secretion. However, in nerve stimulation experiments (Orskov C, Knuthsen S, and Holst J, unpublished studies), we have never been able to measure a co-release of GRP and GLPs, which seems to preclude an association between the two. With respect to somatostatin, we were unable to identify mechanisms that regulate the paracrine somatostatinergic cells restraining GLP secretion (except for the potential paracrine actions of GLP-1 itself). The epithelial somatostatin cells seem to function independently of the extrinsic innervation. Our results show that adrenergic mechanisms strongly inhibit somatostatin secretion but also seem to inhibit GLP secretion, so that diffuse activity in the extrinsic noradrenergic neurons does not cause a stimulated release of GLPs, despite lifting of the somatostatin restraint.

We were concerned about the discrepancy between the amounts of GLP-1 and GLP-2 secreted during somatostatin immunoneutralization; therefore, we performed a chromatographic analysis, which was facilitated by the large amount of immunoreactive material released. Of the glucagon-containing peptides, glicentin predominated; amidated GLP-1 [GLP-1(7—36)amide] and glycine-extended GLP-1 [GLP-1(7—37)] contributed about equally to the total amount of GLP-1 secreted, and GLP-2 occurred predominantly as a single component, identified equally well by side viewing and NH2-terminal assays. Together the two forms of GLP-1 approximately equaled the amount of GLP-2, in agreement with the concept that the two are secreted in equimolar amounts. However, the large contribution of GLP-1(7—37) was unexpected and distinguishes pigs from humans, in whom the amidated form predominates (31). Pigs seem to resemble rats in this respect (26). This observation illustrates the difficulties involved in determining secretion of GLP-1 in vivo, where side-viewing assays, measuring “total” GLP-1, cannot be used because of pancreatic secretion of GLP-1-containing molecules that are picked up by such assays (30). In addition, both NH2-terminally modified and COOH-terminally modified molecular forms are circulating (8, 9), precluding the use of single antibody or sandwich approaches for detection of the total secretory rate of GLP-1. The rate of GLP-2 secretion, estimated using an NH2-terminal assay, may thus provide the best estimate of L cell activity.

Address for reprint requests and other correspondence: J. J. Holst, Dept. of Medical Physiology, University of Copenhagen, the Panum Institute, Blegdamsvej 3, DK-2200 Copenhagen N, Denmark (E-mail: holst@nrlf.ku.dk).

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